Unexpected Functional Divergence of Bat Influenza Virus NS1 Proteins

Turkington, H L ; Juozapaitis, M ; Tsolakos, N ; Corrales-Aguilar, E ; Schwemmle, M ; Hale, B G

Abstract: Recently, two influenza A virus (FLUAV) genomes were identified in Central and South American bats. These sequences exhibit notable divergence from classical FLUAV counterparts, and functionally, bat FLUAV glycoproteins lack canonical receptor binding and destroying activity. Nevertheless, other features that distinguish these viruses from classical FLUAVs have yet to be explored. Here, we studied the viral non-structural protein, NS1, a virulence factor that modulates host signaling to promote efficient propagation. Like all FLUAV NS1 proteins, bat FLUAV NS1s bind double-stranded RNA and act as interferon-antagonists. Unexpectedly, we found that bat FLUAV NS1s are unique in being unable to bind host p85 , a regulatory subunit of the cellular metabolism-regulating enzyme, phosphoinositide 3-kinase (PI3K). Furthermore, neither bat FLUAV NS1 alone, nor infection with a chimeric bat FLUAV, efficiently activates Akt, a PI3K effector. Structure-guided mutagenesis revealed that the bat FLUAV NS1:p85 interaction can be re-engineered (in a strain-specific manner) by changing 2-4 NS1 residues (96L, 99M, 100I and 145T) thereby creating a hydrophobic patch. Notably, ameliorated p85 -binding is insufficient for bat FLUAV NS1 to activate PI3K, and a chimeric bat FLUAV expressing NS1 with engineered hydrophobic patch mutations exhibits cell-type dependent, but species independent, propagation phenotypes. We hypothesize that bat FLUAV hijack of PI3K in the natural bat host has been selected against, perhaps because genes in this metabolic pathway were differentially shaped by evolution to suit unique energy-use strategies of this flying mammal. Our data expand understanding of the enigmatic functional divergence between bat FLUAVs and classical mammalian and avian FLUAVs.IMPORTANCE The potential for novel influenza A viruses to establish infections in humans from animals is a source of continuous concern due to possible severe outbreaks or pandemics. The recent discovery of influenza A-like viruses in bats has raised questions over whether these entities could be a threat to humans. Understanding unique properties of the newly-described bat influenza A-like viruses, such as their mechanisms to infect cells or how they manipulate host functions, is critical to assess their likelihood of causing disease. Here, we characterized the bat influenza A-like virus NS1 protein, a key virulence factor, and found unexpected functional divergence of this protein from counterparts in other influenza A viruses. Our study dissects the molecular changes required by bat influenza A-like virus NS1 to adopt classical influenza A virus properties, and suggests consequences of bat influenza A-like virus infection, potential future evolutionary trajectories, and intriguing virus-host biology in bat species.

DOI: https://doi.org/10.1128/JVI.02097-17

Turkington, H L; Juozapaitis, M; Tsolakos, N; Corrales-Aguilar, E; Schwemmle, M; Hale, B G (2018). Unexpected Functional Divergence of Bat Influenza Virus NS1 Proteins. Journal of Virology, 92(5):e02097-17.
DOI: https://doi.org/10.1128/JVI.02097-17

Unexpected Functional Divergence of Bat Influenza Virus NS1 Proteins

Hannah L. Turkington[a], Mindaugas Juozapaitis[b,c], Nikos Tsolakos[a], Eugenia Corrales-Aguilar[d], Martin Schwemmle[b,c] & Benjamin G. Hale[a#]

Institute of Medical Virology, University of Zurich, Switzerland[a]; Institute of Virology, Medical Center – University of Freiburg, Germany[b]; Faculty of Medicine, University of Freiburg, Germany[c]; Virology-CIET (Research Center for Tropical Diseases), Microbiology, University of Costa Rica, San José, Costa Rica[d]

Running head: An Unexpected Bat FLUAV NS1 Feature

[#]Address correspondence to Benjamin G. Hale, hale.ben@virology.uzh.ch

Abstract word length: 250

Text word length: 4,070 (excluding references and figure legends)

ABSTRACT

Recently, two influenza A virus (FLUAV) genomes were identified in Central and South American bats. These sequences exhibit notable divergence from classical FLUAV counterparts, and functionally, bat FLUAV glycoproteins lack canonical receptor binding and destroying activity. Nevertheless, other features that distinguish these viruses from classical FLUAVs have yet to be explored. Here, we studied the viral non-structural protein, NS1, a virulence factor that modulates host signaling to promote efficient propagation. Like all FLUAV NS1 proteins, bat FLUAV NS1s bind double-stranded RNA and act as interferon-antagonists. Unexpectedly, we found that bat FLUAV NS1s are unique in being unable to bind host p85β, a regulatory subunit of the cellular metabolism-regulating enzyme, phosphoinositide 3-kinase (PI3K). Furthermore, neither bat FLUAV NS1 alone, nor infection with a chimeric bat FLUAV, efficiently activates Akt, a PI3K effector. Structure-guided mutagenesis revealed that the bat FLUAV NS1:p85β interaction can be re-engineered (in a strain-specific manner) by changing 2-4 NS1 residues (96L, 99M, 100I and 145T) thereby creating a hydrophobic patch. Notably, ameliorated p85β-binding is insufficient for bat FLUAV NS1 to activate PI3K, and a chimeric bat FLUAV expressing NS1 with engineered hydrophobic patch mutations exhibits cell-type dependent, but species independent, propagation phenotypes. We hypothesize that bat FLUAV hijack of PI3K in the natural bat host has been selected against, perhaps because genes in this metabolic pathway were differentially shaped by evolution to suit unique energy-use strategies of this flying mammal. Our data expand understanding of the enigmatic functional divergence between bat FLUAVs and classical mammalian and avian FLUAVs.

48 **IMPORTANCE**

49

50 The potential for novel influenza A viruses to establish infections in humans from animals is a
51 source of continuous concern due to possible severe outbreaks or pandemics. The recent
52 discovery of influenza A-like viruses in bats has raised questions over whether these entities
53 could be a threat to humans. Understanding unique properties of the newly-described bat
54 influenza A-like viruses, such as their mechanisms to infect cells or how they manipulate host
55 functions, is critical to assess their likelihood of causing disease. Here, we characterized the
56 bat influenza A-like virus NS1 protein, a key virulence factor, and found unexpected
57 functional divergence of this protein from counterparts in other influenza A viruses. Our study
58 dissects the molecular changes required by bat influenza A-like virus NS1 to adopt classical
59 influenza A virus properties, and suggests consequences of bat influenza A-like virus
60 infection, potential future evolutionary trajectories, and intriguing virus-host biology in bat
61 species.

62

63 **INTRODUCTION**

64

65 In 2012 and 2013, two novel influenza A virus (FLUAV) genomes were discovered by RT-
66 PCR analysis of rectal samples from two species of Guatemalan and Peruvian fruit bats (1, 2).
67 Due to high sequence and evolutionary divergence in multiple genomic segments, as
68 compared with other FLUAVs, these viruses were designated the unique subtypes HL17NL10
69 and HL18NL11 (1-3). Functional characterization of bat FLUAVs and their encoded proteins
70 using reverse genetic or reductionist techniques have since revealed remarkable properties of
71 these viruses [reviewed in (3-6)]. For example, bat FLUAV surface glycoproteins lack
72 canonical receptor binding or destroying features (7-10), and these viruses preferentially enter
73 polarized cells at the basolateral membrane (11). Thus, bat FLUAVs appear to have evolved
74 unique mechanisms for entering host cells that are distinct from classical mammalian and
75 avian FLUAVs. Other features that distinguish bat FLUAVs from other FLUAVs have yet to
76 be explored in detail.

77

78 The precise host-range and zoonotic potential of bat FLUAVs is unknown, although
79 depending upon the experimental system used these viruses can infect cells from an array of
80 species, including bats, humans and dogs (11-13). Reassortment of bat FLUAVs with
81 classical FLUAVs has never been observed in experimental studies (14-16), although a cell-
82 line derived from the bat species *Pteropus alecto* can support replication and reassortment of
83 avian, swine and human FLUAVs (17). In addition, there is evidence that bat species can be
84 naturally infected with classical FLUAVs: in one study 30% of Ghanaian fruit bats analyzed
85 were seropositive for avian H9 FLUAVs (18), and the respiratory and gastro-intestinal tracts
86 of North American little brown bats harbor receptors for both avian and mammalian FLUAVs
87 (19). Thus, these data suggest that bats could represent an additional reservoir for FLUAVs,
88 with the potential to provide either new gene segments or entire viruses that cross the species

89 barrier with unknown consequences. Understanding fundamental properties of the newly-
90 described bat FLUAVs, such as their mechanisms to infect cells of certain species and how
91 they modulate functions of the infected host-cell environment, will be critical to assess their
92 likelihood for causing disease in particular hosts.
93
94 We have focused on the non-structural protein, NS1, of bat FLUAVs, which shares only
95 ~50% sequence identity with other FLUAV NS1 proteins. NS1 is a multifunctional virulence
96 factor that can contribute to virus host-range and virulence (20, 21). A major role of NS1
97 during FLUAV infection is to antagonize the host innate interferon (IFN) system through
98 multiple mechanisms, some of which appear to be strain-dependent (22-31). We and others
99 have recently demonstrated that bat FLUAV NS1 proteins, like classical FLUAVs, harbor a
100 structurally- and functionally- conserved N-terminal double-stranded (ds) RNA-binding
101 domain that is critical for potent IFN-antagonism in human cells (15, 32, 33). The C-terminal
102 effector domain (ED) of bat FLUAV NS1 may play a minor role in supporting IFN-
103 antagonism *in vitro* (15), possibly by promoting NS1 oligomerization (34-37). However, a
104 striking finding is that deletion of the bat FLUAV NS1 ED has minimal impact on viral
105 pathogenesis in a mouse model as compared to deletion of this NS1 domain in a human H1N1
106 FLUAV (15). This suggests unappreciated functional differences between the EDs of bat and
107 classical FLUAVs that remain to be explored. Here, we investigated one such activity of the
108 NS1 ED: modulation of the host intracellular metabolic environment by binding the p85β
109 regulatory subunit of phosphoinositide 3-kinase (PI3K) (38). The NS1 EDs of all classical
110 human and avian FLUAV strains tested to date bind the inter-SH2 (iSH2) domain of host
111 p85β to activate PI3K signaling (38-42). This activation generally benefits classical FLUAV
112 replication, as viruses engineered to express NS1 proteins unable to bind p85β are attenuated
113 for replication *in vitro* and for virulence *in vivo* (38, 43, 44). In this study, we found that bat
114 FLUAV NS1 proteins uniquely lack the ability to bind host p85β and activate PI3K signaling.

We map the bat FLUAV NS1 ED residues responsible for this atypical binding phenotype, and characterize the impact that artificial introduction of this property into a model bat FLUAV has on virus propagation *in vitro*. Our data reveal unexpected functional divergence of bat FLUAV NS1 proteins from classical FLUAV NS1 proteins, and may suggest that specific metabolic conditions in the bat host *in vivo* negate any requirement for NS1 to evolve to engage with host PI3K signaling in these species.

RESULTS

Bat FLUAV NS1 proteins are unable to bind p85β. As part of a larger project to understand the interplay between FLUAV NS1 proteins and host PI3K, we screened a panel of different NS1s from various virus strains for their ability to interact with human p85β, or the related isoform, p85α. To this end, 293T cells were co-transfected with plasmids expressing FLAG-tagged human p85α or p85β, together with plasmids expressing V5-tagged GST or NS1 proteins from representative human, swine, avian and bat strains. Soluble fractions were prepared from cell lysates 48 h post-transfection, and were subjected to immunoprecipitation with an antibody raised against the V5 tag. Western blot analysis of the resulting precipitates revealed that NS1 proteins from human (PR8, H1N1; and Cal/09, pdmH1N1), swine (Sw/Tx/98, H3N2) and avian (Nig/07, H5N1; and Sh/13, H7N9) strains were all capable of interacting specifically with human p85β, but not p85α (**Fig. 1A**). Surprisingly, NS1 proteins from bat FLUAVs (Guat/09, HL17NL10; and Peru/10, HL18NL11) were unable to precipitate either p85α or p85β (**Fig. 1B**). This phenotype has not previously been observed for a naturally-occurring FLUAV NS1 protein, but is akin to the more distantly related influenza B virus (FLUBV) NS1, which is also unable to bind PI3K subunits (45, 46).

Bat FLUAV NS1 proteins do not show a species-specific interaction with p85β. To examine if the interaction between bat FLUAV NS1 proteins and host p85β is species-specific, we generated an expression vector for bat (*Myotis lucifugus*) p85β, and assessed its co-immunoprecipitation with various NS1 proteins. While PR8/NS1 interacted with this bat p85β to an extent similar to human p85β, neither the Guat/09 nor Peru/10 bat FLUAV NS1 proteins were able to precipitate bat p85β (**Fig 2A**). To exclude the possibility that this lack of p85β-binding was due to *M. lucifugus* not being the primary bat host species for bat FLUAVs, we sought to generate expression vectors mimicking p85β from the bat species *Sturnia lilium* (in which the HL17NL10 virus genome was first discovered (1)) and *Carollia perspicillata* (in which individual animals sero-positive for HL18NL11 have been identified (2)). To this end, we cloned and sequenced the region encoding the p85β iSH2 domain from tissue originally derived from *S. lilium* and *C. perspicillata*. As shown in **Fig 2B**, the human and bat p85β iSH2 domains are almost identical, with only 5 residue positions that differ. Three of these differences are conserved in the bat species sequenced (E544, T547 and L550; bat numbering), while two residue positions (605 and 611) differ between the *M. lucifugus* p85β iSH2 domain and the two other bat species (**Fig. 2B**). We therefore substituted these two residue positions in the *M. lucifugus* p85β for the respective residues from *C. perspicillata* (A605S) or *S. lilium* (A605S/D611E), and assessed their abilities to co-precipitate with NS1. However, neither bat p85β variant interacted with the bat FLUAV NS1 proteins, while their interaction with PR8/NS1 was unaffected (**Fig. 2C**). These data suggest that the bat FLUAV NS1 proteins have not specifically evolved to bind bat p85β.

Bat FLUAV is an inefficient activator of PI3K signaling during infection. In contrast to PR8/NS1, and consistent with their inability to bind p85β, isolated expression of the V5-tagged NS1 proteins from Guat/09 or Peru/10 failed to stimulate phosphorylation of the downstream effector of PI3K, Akt (**Fig. 3A**). To assess further the impact of bat FLUAV

infection on host PI3K signaling, we generated recombinant, chimeric bat FLUAVs possessing the six complete internal gene segments of Guat/09 (variant C3; chHL17) and modified HA and NA glycoprotein gene segments from PR8, where the untranslated regions were replaced by the genome ends and packaging sequences of the corresponding segments of Guat/09. Within these viruses, we also re-engineered the NS segment to allow expression of different V5-tagged NS1 proteins (from PR8, Guat/09 or Peru/10) separated from Guat/09 NEP by a 2A sequence (**Fig. 3B**). Immunoprecipitations from infected cell lysates revealed that endogenous p85β could be co-precipitated with V5-PR8/NS1, but not with the V5-tagged NS1 proteins derived from Guat/09 or Peru/10 (**Fig. 3C**). In addition, we observed that only the chimeric bat FLUAV expressing V5-tagged PR8/NS1 efficiently lead to increased phosphorylation of Akt during infection (**Figure 3D**). These infection data indicate that additional bat FLUAV internal gene segment products are unable to compensate bat FLUAV NS1 for either binding host p85β or activating PI3K.

A small number of amino acid changes can establish an interaction between bat FLUAV NS1 and p85β. We analyzed the crystal structure of the PR8/NS1 ED in complex with the p85β iSH2 domain, taking into account recent mutagenesis data (**Fig. 4A**) (41, 47). Of the NS1 residues present at this interface, 6 were selected for substitution analysis in the bat FLUAV NS1 proteins based on their divergence from residues in NS1 proteins with the ability to bind p85β (**Figs. 4A** and **4B**). Single amino acid substitutions at these 6 ED residues in Guat/09 NS1 (Q96L, T99M, I100S, R145T, N163S and S166P; Guat/09 NS1 numbering), did not result in the establishment of human p85β binding (**Fig. 4C**). However, combining the amino acid substitutions Q96L and T99M resulted in partial p85β binding (**Fig. 4D**). The interaction between Guat/09 NS1 and human p85β could be enhanced further when the additional R145T substitution was introduced (**Fig. 4D**). Substitutions at other positions (particularly I100S) proved to be disruptive to the interaction (**Fig. 4D**). From these data, we

193 infer that creation of a hydrophobic patch around positions 96, 99 and 100 in the Guat/09 NS1
194 ED is essential for determining the interaction with p85β. Largely similar, but distinct, results
195 were obtained for substitution analysis of the Peru/10 NS1 protein: single amino acid
196 substitution of V96L, T99M or R145T, as well as double substitution of V96L and T99M, did
197 not establish an interaction with human p85β (**Figs. 5A** and **5B**). In fact, only when N100I
198 was combined with double substitution of V96L and T99M could some weak p85β binding be
199 observed, which was strongly enhanced by R145T (**Fig. 5B**). Notably, mutant Guat/09 and
200 Peru/10 NS1 constructs generated to possess optimal p85β binding also exhibited the ability
201 to interact with bat p85β (**Fig. 5C**).
202
203 **A chimeric bat FLUAV expressing an engineered p85β-binding NS1 protein exhibits**
204 **cell-type dependent, but species-independent, propagation phenotypes.** Using reverse
205 genetics, we generated chimeric bat FLUAVs as before (chHL17), but possessing authentic
206 Guat/09 NS segments expressing either wild-type NS1 (WT) or NS1-Q96L/T99M (2x), which
207 possesses p85β-binding ability with a minimal number of substitutions. Strikingly, we
208 observed that chHL17-2x exhibited an altered plaque phenotype and markedly enhanced
209 propagation capability (>100-fold higher peak titers) in canine MDCK cells (**Figs. 6A** and
210 **6B**). Enhanced propagation of the chHL17-2x virus was also observed, to a lesser extent, in
211 human HAP-1 cells (**Fig. 6C**). However, such a phenotype was not found in other cell-types:
212 the chHL17-2x virus exhibited slower replication kinetics in human A549 cells, and a subtle
213 attenuation in bat EidNi/41 cells (**Figs. 6D** and **6E**). These data indicate that the Q96L/T99M
214 substitutions in NS1 impact tissue-culture replication of the chimeric bat FLUAV in a cell-
215 type dependent, but species-independent, manner.
216
217 **Role of p85β and PI3K activation in the phenotype of a chimeric bat FLUAV expressing**
218 **NS1-Q96L/T99M.** We hypothesized that the engineered binding of Guat/09 NS1-

Q96L/T99M to p85β, and potential activation of PI3K signaling, was responsible for modulating propagation of the chHL17-2x virus. However, similar to expression of the wild-type V5-tagged NS1 protein from Guat/09, expression of V5-Guat/09 NS1-Q96L/T99M failed to stimulate Akt phosphorylation (**Fig. 7A**), an observation supporting previous data that other NS1 determinants beyond efficient p85β binding can critically impact PI3K activation (41, 43, 48). In addition, we assessed propagation of the chHL17-2x virus in human HAP-1 cells that had been genetically-engineered to lack p85β expression (**Fig. 7B**). Notably, the chHL17-2x virus also exhibited slightly enhanced propagation in this p85β knock-out HAP-1 cell-line (**Fig. 7C**), which was similar to the enhancement previously observed in wild-type HAP-1 cells (**Figs. 6C and 7D**). Overall, these data suggest that, while the Q96L/T99M substitutions in Guat/09 NS1 permit host p85β-binding, they are not sufficient for this NS1 to activate host PI3K signaling. Furthermore, these NS1 substitutions may impact propagation of the chimeric bat FLUAV, at least in some host substrates, in a p85β/PI3K-independent manner.

DISCUSSION

Functional characterization of proteins encoded by the novel bat FLUAV sequences has so far revealed some remarkable properties, most notably that the HA-like protein does not bind canonical FLUAV sialic acid receptors, and the NA-like protein lacks neuraminidase activity (7-10). Herein, we describe an additional feature of classical mammalian and avian FLUAVs that is missing in their bat-derived counterparts: the multifunctional bat FLUAV NS1 virulence factor is unable to bind host p85β or activate PI3K signaling. This property seems important for the efficient replication of many classical FLUAV strains in tissue-culture and *in vivo* (38, 43, 44, 47). Using a structure-guided approach, we found that p85β-binding could be engineered into the bat FLUAV NS1 proteins with a minimum of only 2 amino-acid

substitutions (Q96L/T99M), and that a chimeric bat FLUAV expressing NS1 with these substitutions had improved propagation kinetics in some tissue-culture systems (canine MDCK and human HAP-1), but a seemingly attenuated phenotype in others (human A549 and bat EidNi/41). Notably, this disparate impact did not correlate with host-species, as two different human cell-lines were identified in which the outcome of infection was opposite. Therefore, cell-type (and thus unknown differential gene/protein expression profiles or polymorphisms) likely determine the phenotypic result. Interestingly, the propagation phenotype in human HAP-1 cells of the chimeric bat FLUAV expressing NS1-Q96L/T99M was not diminished in an isogenic cell-line completely lacking p85β expression, suggesting that the amino-acid substitutions used to permit p85β-binding by NS1 also modify an additional property of this multifunctional viral protein that has yet to be identified. This will certainly be an interesting area to explore in the future, and may impact our understanding of other classical influenza A viruses and the biological consequences of certain NS1 polymorphisms that they harbor. Such observations will also have implications when assessing potential virological consequences of the p85β-binding property arising naturally in bat FLUAVs.

Engineering p85β-binding into the bat FLUAV NS1 protein did not result in detectable activation of PI3K signaling, indicating that additional regions of NS1 are essential for this function. Indeed, while p85β-binding is necessary for NS1-mediated PI3K activation (38), our data suggest that it is not sufficient, an observation consistent with previous work (41, 48). Glutamic acid residues at human FLUAV NS1 positions 96 and 97 (equivalent to bat FLUAV NS1 positions 97 and 98) do not interact directly with p85β, but have been proposed to form part of an ‘activating interface’ with the p110 catalytic subunit of the p85β-containing PI3K heterodimer (41). Although these are the only non-p85β-interface residues so far implicated in NS1-activated PI3K signaling, they are conserved in the bat FLUAV NS1 proteins, meaning

271 that another unidentified region of classical FLUAV NS1 proteins must be responsible for
272 ensuring PI3K activation. Experiments to uncover this specific region are currently underway,
273 but it is therefore apparent that more than the 2-4 amino-acid substitutions required for p85β-
274 binding would be necessary to allow bat FLUAV NS1 proteins to fully acquire PI3K
275 activation potential, constituting a relatively high genetic barrier to natural evolution of this
276 property.
277
278 Our studies suggest that interactions between NS1 and p85β are not species-specific: bat
279 FLUAV NS1 proteins do not bind bat p85β. Thus, an open question is why bat FLUAVs have
280 not retained (or evolved) this property like classical mammalian and avian FLUAVs? One
281 hypothesis for this observation is that the natural host environment encountered *in vivo* by bat
282 FLUAVs is in a particular metabolic state such that viral augmentation of PI3K signaling
283 would not alter host physiology. Indeed, recent genome sequencing and analysis studies have
284 begun to uncover major differences between bats and other species, and have linked
285 specialized adaptations of bats (e.g. flying ability, longevity, hibernation and echolocation)
286 with specific adaptive evolution of energy metabolism genes. Such genes include those
287 involved in oxidative phosphorylation (49), insulin/growth factor receptor signaling (50), and
288 DNA damage repair and apoptosis (51), pathways otherwise known to be regulated by PI3K
289 in many mammalian systems (52, 53). Possible links between positive selection in bat
290 metabolic genes and decreased sensitivity to PI3K-mediated regulation need to be validated
291 experimentally, but could account for bat FLUAV NS1 proteins adopting a different
292 evolutionary path to those from their classical FLUAV counterparts in other species. Such
293 functional divergence of bat FLUAV NS1 proteins underscores the uniqueness of these
294 newly-discovered viruses, and suggests new and intriguing virus-host interaction biology in
295 bat species that remains to be explored.

296 **MATERIALS AND METHODS**

297

298 **Cells.** MDCK (canine), 293T (human), A549 (human), 1321N1 (human), EidNi/41 (bat;
299 *Eidolon helvum*) (54) and CarperAEC.B-3 (bat; *Carollia perspicillata*) (55) cells were
300 cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL
301 penicillin and 100 µg/mL streptomycin (Gibco Life Technologies). HAP-1 cells (Horizon
302 Discovery, Austria) were cultured in IMDM supplemented with the same additives as well as
303 200µM GlutaMax™ (Gibco Life Technologies). The HAP-1 cell-line engineered by
304 CRISPR/Cas9 to have a 2bp deletion in the *PIK3R2* gene (encoding p85β) was purchased
305 from Horizon Discovery, Austria (catalogue ID: HZGHC003292c006). All cells were
306 maintained at 37˚C with 5% CO_2.

307

308 **Expression plasmids.** The NS1 cDNA sequences from A/Swine/Texas/4199-2/98 (Sw/Tx/98,
309 H3N2), A/Nigeria/OG10/2007 (Nig/07, H5N1), A/Shanghai/2/S1078/2013 (Sh/13, H7N9),
310 A/little yellow shouldered bat/Guatemala/153/2009 (Guat/09, HL17NL10) and A/flat-faced
311 bat/Peru/033/2010 (Peru/10, HL18NL11) were PCR amplified from existing plasmids and
312 ligated in-frame with an N-terminal V5-tag into modified pLVX-IRES-ZsGreen1 or pLVX-
313 IRES-Puro plasmids (Clontech, USA). Similar plasmids expressing glutathione S-transferase
314 (GST) and NS1 sequences from A/Puerto Rico/8/1934 (PR8, H1N1) or A/California/04/09
315 (Cal09, pdmH1N1) have been described previously (47). All NS1-encoding cDNAs contained
316 silent mutations in the splice acceptor site to prevent expression of NEP. Human p85α and
317 p85β cDNA sequences from existing plasmids, or the bat (*Myotis lucifugus*) p85β cDNA
318 sequence generated using the GeneArt gene synthesis service (Thermo Fisher), were PCR
319 amplified and ligated in-frame into p3xFLAG-CMV-7.1 (Sigma-Aldrich) so as to express
320 with N-terminal FLAG tags. Two step overlap PCR or Quikchange II XL (Agilent
321 Technologies, Switzerland) was used to introduce site-directed mutations into cDNAs as

322 required. The identity of all constructs was confirmed by sequencing. All plasmid
323 transfections were performed using Fugene HD (Promega) at 1:3 DNA:transfection reagent
324 ratio. As appropriate, generation of lentiviruses and production of stable cell-lines
325 constitutively expressing proteins of interest were carried out as previously described (38).
326
327 **Virus reverse genetics.** Plasmids for rescuing chimeric, recombinant HL17NL10 virus
328 (variant C3: PA-S550R and M2-N31S/T70A) were described previously (14). pHW2000
329 rescue plasmids comprising the genome ends and packaging sequences of HL17NL10 and the
330 PR8 HA or NA glycoprotein ORFs were generated as described (15). Briefly, the untranslated
331 regions of PR8 HA and NA genome segments were replaced with the genome ends and
332 packaging sequences of the corresponding segments of HL17NL10 by assembly PCR.
333 Plasmids encoding NS segments where NS1 and NEP ORFs were separated by the 2A self-
334 cleaving peptide of porcine teschovirus (allowing N-terminal V5-tagging of NS1) were
335 generated by assembly PCR according to a previous strategy (56). All generated NS chimeras
336 contained silent mutations in the splice acceptor and donor sites of the ORFs to prevent
337 aberrant splicing, and duplicated genome packaging sequences were introduced flanking the
338 NS1 ORF to avoid packaging defects. Quikchange II XL (Agilent Technologies, Switzerland)
339 was used to introduce site-directed mutations into cDNAs as required. For rescues, $6x10^5$
340 293T cells were seeded in 6-well plates and co-transfected 24h later with the 8 pDZ- or
341 pHW2000- based plasmids. Twenty-four hours post-transfection, cells were washed once in
342 sterile phosphate-buffered saline (PBS) and $3x10^5$ MDCK cells were added in DMEM
343 supplemented with 1µg/mL tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated
344 trypsin (Sigma-Aldrich, MO). Forty-eight hours later, supernatants were harvested, viruses
345 plaque-purified, and virus stocks grown and titrated using standard methods in MDCK cells.
346 RNA was extracted from stock aliquots using the ReliaPrep™ RNA Cell Miniprep System

(Promega), and the NS genomic segments of each virus were fully sequenced after segment-specific reverse transcription-PCR (RT-PCR) to ensure absence of undesired mutations.

Virus infections. For all infections, viruses were diluted in PBS supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco Life Technologies), 0.3% BSA (Sigma-Aldrich) and 1 mM Ca^{2+}/Mg^{2+}. For HAP-1 cells, cells were seeded onto poly-L-lysine (Sigma-Aldrich) coated plates and infected 24 h later at the indicated multiplicity of infection (MOI) for 1 h at 37°C. Following infection, cells were washed three times and overlaid with FBS-free IMDM. For serum-starvation experiments, cells were washed 3 times with FBS-free IMDM and incubated overnight prior to infection.

For virus growth analyses, cells in 12-well plates were infected with the indicated virus at the indicated MOI. Following infection at 37°C for 1 h, cells were washed three times with PBS, and overlaid with DMEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco Life Technologies) and 0.5 µg/mL TPCK-treated trypsin (Sigma-Aldrich). Supernatant samples were harvested at the indicated timepoints and stored at -80°C prior to titration by standard plaque assay.

Sequencing of p85β (*PIK3R2*) iSH2 domains from bat species. Bats (*S. lilium)* were captured with mist nets. Specimens were collected and processed after approval of the Institutional Committee of Care and Use of Animals (IACUC) of the University of Costa Rica (CICUA-36-13) according to national guidelines for animal caring described in the Costa Rica National Law for Animal Welfare 7451. For sequencing, $2.5x10^5$ CarperAEC.B-3 cells seeded in 12-well plates, or an equivalent amount of cellular material from *S. lilium*, were lysed, and RNA extracted, using the ReliaPrep™ RNA Cell Miniprep System (Promega). RT-

372 PCR products were generated, and directly sequenced, using specific primers annealing to
373 conserved regions of *PIK3R2* flanking the sequence encoding the p85β iSH2 domain.
374
375 **Immunoprecipitations.** Transfected (2 µg total DNA, 48 h in 293T) or infected (MOI 5
376 PFU/cell, 8 h in HAP-1) cells in 25cm^2 flasks or 6-well plates were lysed on ice in 1mL of
377 20mM Tris-HCl (pH 7.8), 5mM EDTA, 0.5% (v/v) NP40, and 650mM NaCl, supplemented
378 with a cOmplete^{TM} mini protease inhibitor tablet (Roche, Switzerland). Lysates were clarified
379 using a 29G (0.33mm) needle and centrifugation at 13,000 rpm for 40 mins at 4°C. Soluble
380 fractions were then incubated with 1 µg of anti-V5 antibody (Bio-Rad) for 2 hours at 4°C
381 prior to further incubation overnight at 4°C with 12.5 µL protein G sepharose beads (Sigma-
382 Aldrich). Following extensive washing, remaining proteins were dissociated from the beads
383 using urea disruption buffer (6M urea, 1M β-mercaptoethanol, 4% SDS) and heating at 95°C
384 for 10 min. Samples were stored at -20˚C until analysis by SDS-PAGE and western blotting.
385
386 **Akt phosphorylation assays.** 1321N1 cells stably expressing the indicated V5-tagged protein
387 were seeded into 12-well plates and serum-starved the following day for 1 h. Cell lysates
388 were harvested in urea disruption buffer and stored at -20˚C until analysis by SDS-PAGE and
389 western blotting.
390
391 **SDS-PAGE and western blotting.** Samples were sonicated to shear nucleic acids and then
392 boiled for 5 min. Polypeptides were resolved by SDS-PAGE on NuPAGE 4-12% Bis-Tris
393 protein gels (ThermoFisher), followed by transfer to nitrocellulose membranes (GE
394 Healthcare Life Sciences). Proteins were detected by western blotting using the following
395 primary antibodies: mouse anti-V5 (Bio-Rad, MCA1360); mouse anti-FLAG (Sigma-Aldrich,
396 #F3165); mouse anti-p85β (T15; AbD Serotech, MCA1170G); rabbit anti-actin (Sigma-
397 Aldrich, #A2103); rabbit anti-Akt (Cell Signaling Technology, #4691); rabbit anti-pAkt

(S473) (Cell Signaling Technology, #4060); rabbit anti-NS1 (1-73) (57) (kindly provided by Adolfo García-Sastre, Icahn School of Medicine at Mount Sinai, New York, USA); and rabbit anti-NP (kindly provided by Silke Stertz, University of Zurich, Switzerland). Secondary antibodies were fluorochrome-conjugated: anti-mouse (ThermoFisher Scientific, #SA5-10176) and anti-rabbit (ThermoFisher Scientific, #SA5-10036). A LI-COR Odyssey Fc scanner was used for detection.

Structural analyses. Structural representations were visualized using the appropriate PDB file and PyMOL (58).

FUNDING INFORMATION

This work was supported by the Swiss National Science Foundation (grant 31003A_159993 to BGH), a Forschungskredit of the University of Zurich (grant FK-16-030 to HLT), and by the Deutsche Forschungsgemeinschaft (SCHW 632/17-1 to MS). The funders had no role in study design, data collection, data interpretation, or the decision to submit the work for publication.

ACKNOWLEDGEMENTS

We thank Silke Stertz and Antonio M. Lopes (University of Zurich, Switzerland), Adolfo García-Sastre (Icahn School of Medicine at Mount Sinai, USA) and Marcel Müller (University of Bonn, Germany) for sharing material/reagents.

422 **FIGURE LEGENDS**

423

424 **Figure 1. Strain-specific interaction of FLUAV NS1 proteins with human p85β.** 293T
425 cells were co-transfected for 48 h with plasmids expressing the indicated V5-tagged NS1
426 protein (or GST) and FLAG-tagged human p85α or p85β. Following cell lysis, clarification
427 and anti-V5 immunoprecipitation, soluble (input) and pull-down (IP:α-V5) fractions were
428 analyzed by SDS-PAGE and western blot. (**A**) Interaction studies of human (PR8, Cal/09),
429 swine (Sw/Tx/98) and avian (Nig/07, Sh/13) FLUAV NS1 proteins. (**B**) Interaction studies of
430 bat (Guat/09, Peru/10) FLUAV NS1 proteins. Data are representative of 3 independent
431 experiments.

432

433 **Figure 2. Bat FLUAV NS1 proteins do not show a species-specific interaction with p85β.**
434 (**A**) 293T cells were co-transfected for 48 h with plasmids expressing the indicated V5-tagged
435 NS1 protein (or GST) and FLAG-tagged human (*Homo sapiens*, *H.s.*) or bat (*Myotis*
436 *lucifugus*, *M.l.*) p85β. Following cell lysis, clarification and anti-V5 immunoprecipitation,
437 soluble (input) and pull-down (IP:α-V5) fractions were analyzed by SDS-PAGE and western
438 blot. (**B**) Sequence alignment of iSH2 domains from human and bat p85β. Human (*H.*
439 *sapiens)*, little yellow shouldered bat (*S. lilium)*, little brown bat (*M. lucifugus*) and Seba's
440 short-tailed bat (*C. perspicillata*) p85β iSH2 domain amino acid sequences (residues 496-614)
441 were aligned. Human/bat differences are highlighted with boxes, shading indicates residues
442 differing between bat species. (**C**) Experiment performed as in (A), except using the indicated
443 bat p85β mutants. For (**A**) and (**C**), data are representative of 3 independent experiments.

444

445 **Figure 3. Bat FLUAV is an inefficient activator of PI3K signaling during infection.** (**A**)
446 Serum-starved 1321N1 cells stably expressing the indicated V5-tagged proteins were lysed,
447 and levels of the indicated proteins were analyzed by SDS-PAGE and western blot. (**B**)

448 Schematic representation of mRNAs generated by wild-type (*upper*) or engineered (*lower*)
449 NS segments. SD, splice donor; SA, splice acceptor; 2A, 2A sequence from porcine
450 teschovirus. (**C**) HAP-1 cells were infected for 8 h with chHL17 viruses expressing the
451 indicated V5-tagged NS1 proteins at a MOI of 5 PFU/cell. Following cell lysis, clarification
452 and anti-V5 immunoprecipitation, soluble (input) and pull-down (IP:α-V5) fractions were
453 analyzed by SDS-PAGE and western blot. (**D**) Serum-starved HAP-1 cells were infected with
454 chHL17 viruses expressing the indicated V5-tagged NS1 proteins at a MOI of 5 PFU/cell.
455 Cells were lysed at 3, 6 and 9 hours post-infection and levels of the indicated proteins were
456 analyzed by SDS-PAGE and western blot. For (**A**), (**C**) and (**D**), data are representative of at
457 least 2 independent experiments.
458
459 **Figure 4. Engineering the bat FLUAV (Guat/09) NS1 protein to bind p85β.** (**A**) Crystal
460 structure of the PR8/NS1 ED in complex with part of the p85β iSH2 domain. The NS1 ED is
461 shown in gray, with p85β contact residues highlighted in yellow. The p85β iSH2 domain is
462 colored blue. The six NS1 amino acid residues chosen for mutagenesis studies (see **B**,
463 Guat/09 numbering) are highlighted in red. Figure generated using PyMOL with PDB ID:
464 3L4Q. (**B**) Comparison of key NS1 ED residues between human (PR8, Cal09), swine
465 (Sw/Tx/98), avian (Nig/07, Sh/13) and bat (Guat/09, Peru/10) NS1 proteins. #Guat/09
466 numbering. (**C**-**D**) 293T cells were co-transfected for 48 h with plasmids expressing the
467 indicated V5-tagged NS1 protein (or GST) and FLAG-tagged human p85β. Following cell
468 lysis, clarification and anti-V5 immunoprecipitation, soluble (input) and pull-down (IP:α-V5)
469 fractions were analyzed by SDS-PAGE and western blot. Data are representative of at least 2
470 independent experiments.
471
472 **Figure 5. Engineering the bat FLUAV (Peru/10) NS1 protein to bind p85β.** (**A**-**B**) 293T
473 cells were co-transfected for 48 h with plasmids expressing the indicated V5-tagged NS1

474 protein (or GST) and FLAG-tagged human p85β. Following cell lysis, clarification and anti-
475 V5 immunoprecipitation, soluble (input) and pull-down (IP:α-V5) fractions were analyzed by
476 SDS-PAGE and western blot. (**C**) Experiment performed as in (A), except using the indicated
477 bat p85β mutants. Guat/09 3x = Q96L/T99M/R145T; Peru/10 4x =
478 V96L/T99M/N100I/R145T. For all panels, data are representative of at least 2 independent
479 experiments.
480
481 **Figure 6. Characterization of a chimeric bat FLUAV expressing an engineered p85β-**
482 **binding NS1 protein.** (**A**) Plaque phenotype and (**B**) multicycle growth analysis of chHL17-
483 WT and chHL17-2x viruses in MDCK cells. (**C-E**) Multicycle growth analysis of chHL17-
484 WT and chHL17-2x viruses in HAP-1 (**C**), A549 (**D**) and EidNi/41 (**E**) cells. For all growth
485 curves, data represent mean values from 3 independent experiments (± SD). Significance was
486 calculated using the Student's t-test comparing cHL17-WT and chHL17-2x virus titers at each
487 time point (** $p<0.01$, * $p<0.05$).
488
489 **Figure 7. Role of p85β and PI3K activation in the phenotype of a chimeric bat FLUAV**
490 **expressing NS1-Q96L/T99M.** (**A**) Serum-starved 1321N1 cells stably expressing the
491 indicated V5-tagged proteins were lysed, and levels of the indicated proteins were analyzed
492 by SDS-PAGE and western blot. (**B**) Western blot analysis of wild-type or *PIK3R2* (p85β)
493 knock-out HAP-1 cells. For (**A**) and (**B**), data are representative of at least 2 independent
494 experiments. (**C**) Multicycle growth analysis of chHL17-WT and chHL17-2x viruses in
495 *PIK3R2* (p85β) knock-out HAP-1 cells. Data represent mean values from 3 independent
496 experiments (± SD). **(D)** Ratios of chHL17-2x to chHL17-WT propagation titers (represented
497 as area under the curve (AUC) values) in wild-type and *PIK3R2* (p85β) knock-out HAP-1
498 cells (original data from Figs. 6C and 7C). For (C) and (D), significance was calculated using

the Student's t-test comparing chHL17-WT and chHL17-2x virus titers at each time point (* $p<0.05$), or AUC ratios in each cell-line (ns, non significant).

REFERENCES

1. **Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO.** 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A **109:**4269-4274.

2. **Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO.** 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog **9:**e1003657.

3. **Ma W, Garcia-Sastre A, Schwemmle M.** 2015. Expected and Unexpected Features of the Newly Discovered Bat Influenza A-like Viruses. PLoS Pathog **11:**e1004819.

4. **Brunotte L, Beer M, Horie M, Schwemmle M.** 2016. Chiropteran influenza viruses: flu from bats or a relic from the past? Curr Opin Virol **16:**114-119.

5. **Wu Y, Wu Y, Tefsen B, Shi Y, Gao GF.** 2014. Bat-derived influenza-like viruses H17N10 and H18N11. Trends Microbiol **22:**183-191.

6. **Ciminski K, Thamamongood T, Zimmer G, Schwemmle M.** 2017. Novel insights into bat influenza A viruses. J Gen Virol **98:**2393-2400.

7. **Li Q, Sun X, Li Z, Liu Y, Vavricka CJ, Qi J, Gao GF.** 2012. Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. Proc Natl Acad Sci U S A **109:**18897-18902.

8. **Sun X, Shi Y, Lu X, He J, Gao F, Yan J, Qi J, Gao GF.** 2013. Bat-derived influenza hemagglutinin H17 does not bind canonical avian or human receptors and most likely uses a unique entry mechanism. Cell Rep **3:**769-778.

9. **Zhu X, Yang H, Guo Z, Yu W, Carney PJ, Li Y, Chen LM, Paulson JC, Donis RO, Tong S, Stevens J, Wilson IA.** 2012. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. Proc Natl Acad Sci U S A **109:**18903-18908.

10. **Zhu X, Yu W, McBride R, Li Y, Chen LM, Donis RO, Tong S, Paulson JC, Wilson IA.** 2013. Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. Proc Natl Acad Sci U S A **110:**1458-1463.

11. **Moreira EA, Locher S, Kolesnikova L, Bolte H, Aydillo T, Garcia-Sastre A, Schwemmle M, Zimmer G.** 2016. Synthetically derived bat influenza A-like viruses reveal a cell type- but not species-specific tropism. Proc Natl Acad Sci U S A doi:10.1073/pnas.1608821113.

12. **Hoffmann M, Kruger N, Zmora P, Wrensch F, Herrler G, Pohlmann S.** 2016. The Hemagglutinin of Bat-Associated Influenza Viruses Is Activated by TMPRSS2 for pH-Dependent Entry into Bat but Not Human Cells. PLoS One **11:**e0152134.

13. **Maruyama J, Nao N, Miyamoto H, Maeda K, Ogawa H, Yoshida R, Igarashi M, Takada A.** 2016. Characterization of the glycoproteins of bat-derived influenza viruses. Virology **488:**43-50.

14. **Juozapaitis M, Aguiar Moreira E, Mena I, Giese S, Riegger D, Pohlmann A, Hoper D, Zimmer G, Beer M, Garcia-Sastre A, Schwemmle M.** 2014. An infectious bat-derived chimeric influenza virus harbouring the entry machinery of an influenza A virus. Nat Commun **5:**4448.

15. **Zhou B, Ma J, Liu Q, Bawa B, Wang W, Shabman RS, Duff M, Lee J, Lang Y, Cao N, Nagy A, Lin X, Stockwell TB, Richt JA, Wentworth DE, Ma W.** 2014. Characterization of uncultivable bat influenza virus using a replicative synthetic virus. PLoS Pathog **10:**e1004420.

16. **Yang J, Lee J, Ma J, Lang Y, Nietfeld J, Li Y, Duff M, Li Y, Yang Y, Liu H, Zhou B, Wentworth DE, Richt JA, Li Z, Ma W.** 2017. Pathogenicity of modified bat influenza virus with different M genes and its reassortment potential with swine influenza A virus. J Gen Virol **98:**577-584.

17. **Dlugolenski D, Jones L, Tompkins SM, Crameri G, Wang LF, Tripp RA.** 2013. Bat cells from Pteropus alecto are susceptible to influenza A virus infection and reassortment. Influenza and Other Respiratory Viruses **7:**900-903.

18. **Freidl GS, Binger T, Muller MA, de Bruin E, van Beek J, Corman VM, Rasche A, Drexler JF, Sylverken A, Oppong SK, Adu-Sarkodie Y, Tschapka M, Cottontail VM, Drosten C, Koopmans M.** 2015. Serological evidence of influenza A viruses in frugivorous bats from Africa. PLoS One **10:**e0127035.

19. **Chothe SK, Bhushan G, Nissly RH, Yeh YT, Brown J, Turner G, Fisher J, Sewall BJ, Reeder DM, Terrones M, Jayarao BM, Kuchipudi SV.** 2017. Avian and human influenza virus compatible sialic acid receptors in little brown bats. Sci Rep **7:**660.

20. **Noronha JM, Liu M, Squires RB, Pickett BE, Hale BG, Air GM, Galloway SE, Takimoto T, Schmolke M, Hunt V, Klem E, Garcia-Sastre A, McGee M, Scheuermann RH.** 2012. Influenza virus sequence feature variant type analysis: evidence of a role for NS1 in influenza virus host range restriction. J Virol **86:**5857-5866.

21. **Hale BG.** 2014. Conformational plasticity of the influenza A virus NS1 protein. J Gen Virol **95:**2099-2105.

22. **Krug RM.** 2015. Functions of the influenza A virus NS1 protein in antiviral defense. Curr Opin Virol **12:**1-6.

23. **Rajsbaum R, Albrecht RA, Wang MK, Maharaj NP, Versteeg GA, Nistal-Villan E, Garcia-Sastre A, Gack MU.** 2012. Species-specific inhibition of RIG-I

ubiquitination and IFN induction by the influenza A virus NS1 protein. PLoS Pathog **8:**e1003059.

24. **Ayllon J, Domingues P, Rajsbaum R, Miorin L, Schmolke M, Hale BG, Garcia-Sastre A.** 2014. A Single Amino Acid Substitution in the Novel H7N9 Influenza A Virus NS1 Protein Increases CPSF30 Binding and Virulence. J Virol **88:**12146-12151.

25. **Hale BG, Steel J, Medina RA, Manicassamy B, Ye J, Hickman D, Hai R, Schmolke M, Lowen AC, Perez DR, Garcia-Sastre A.** 2010. Inefficient control of host gene expression by the 2009 pandemic H1N1 influenza A virus NS1 protein. J Virol **84:**6909-6922.

26. **Kochs G, Garcia-Sastre A, Martinez-Sobrido L.** 2007. Multiple anti-interferon actions of the influenza A virus NS1 protein. J Virol **81:**7011-7021.

27. **Twu KY, Kuo RL, Marklund J, Krug RM.** 2007. The H5N1 influenza virus NS genes selected after 1998 enhance virus replication in mammalian cells. J Virol **81:**8112-8121.

28. **Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, Garcia-Sastre A.** 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe **5:**439-449.

29. **Marazzi I, Ho JS, Kim J, Manicassamy B, Dewell S, Albrecht RA, Seibert CW, Schaefer U, Jeffrey KL, Prinjha RK, Lee K, Garcia-Sastre A, Roeder RG, Tarakhovsky A.** 2012. Suppression of the antiviral response by an influenza histone mimic. Nature **483:**428-433.

30. **Kuo RL, Zhao C, Malur M, Krug RM.** 2010. Influenza A virus strains that circulate in humans differ in the ability of their NS1 proteins to block the activation of IRF3 and interferon-beta transcription. Virology **408:**146-158.

31. **Noah DL, Twu KY, Krug RM.** 2003. Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAS. Virology **307:**386-395.

32. **Turkington HL, Juozapaitis M, Kerry PS, Aydillo T, Ayllon J, Garcia-Sastre A, Schwemmle M, Hale BG.** 2015. Novel Bat Influenza Virus NS1 Proteins Bind Double-Stranded RNA and Antagonize Host Innate Immunity. J Virol **89:**10696-10701.

33. **Zhao X, Tefsen B, Li Y, Qi J, Lu G, Shi Y, Yan J, Xiao H, Gao GF.** 2016. The NS1 gene from bat-derived influenza-like virus H17N10 can be rescued in influenza A PR8 backbone. J Gen Virol **97:**1797-1806.

34. **Hale BG, Barclay WS, Randall RE, Russell RJ.** 2008. Structure of an avian influenza A virus NS1 protein effector domain. Virology **378:**1-5.

35. **Kerry PS, Ayllon J, Taylor MA, Hass C, Lewis A, Garcia-Sastre A, Randall RE, Hale BG, Russell RJ.** 2011. A transient homotypic interaction model for the influenza A virus NS1 protein effector domain. PLoS One **6:**e17946.

36. **Ayllon J, Russell RJ, Garcia-Sastre A, Hale BG.** 2012. Contribution of NS1 effector domain dimerization to influenza A virus replication and virulence. J Virol **86:**13095-13098.

37. **Aramini JM, Ma LC, Zhou L, Schauder CM, Hamilton K, Amer BR, Mack TR, Lee HW, Ciccosanti CT, Zhao L, Xiao R, Krug RM, Montelione GT.** 2011. Dimer interface of the effector domain of non-structural protein 1 from influenza A virus: an interface with multiple functions. J Biol Chem **286:**26050-26060.

38. **Hale BG, Jackson D, Chen YH, Lamb RA, Randall RE.** 2006. Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. Proc Natl Acad Sci U S A **103:**14194-14199.

39. **Hale BG, Batty IH, Downes CP, Randall RE.** 2008. Binding of influenza A virus NS1 protein to the inter-SH2 domain of p85 suggests a novel mechanism for phosphoinositide 3-kinase activation. J Biol Chem **283:**1372-1380.

40. **Fan S, Macken CA, Li C, Ozawa M, Goto H, Iswahyudi NF, Nidom CA, Chen H, Neumann G, Kawaoka Y.** 2013. Synergistic effect of the PDZ and p85beta-binding domains of the NS1 protein on virulence of an avian H5N1 influenza A virus. J Virol **87:**4861-4871.

41. **Hale BG, Kerry PS, Jackson D, Precious BL, Gray A, Killip MJ, Randall RE, Russell RJ.** 2010. Structural insights into phosphoinositide 3-kinase activation by the influenza A virus NS1 protein. Proc Natl Acad Sci U S A **107:**1954-1959.

42. **Pichlmair A, Kandasamy K, Alvisi G, Mulhern O, Sacco R, Habjan M, Binder M, Stefanovic A, Eberle CA, Goncalves A, Burckstummer T, Muller AC, Fauster A, Holze C, Lindsten K, Goodbourn S, Kochs G, Weber F, Bartenschlager R, Bowie AG, Bennett KL, Colinge J, Superti-Furga G.** 2012. Viral immune modulators perturb the human molecular network by common and unique strategies. Nature **487:**486-490.

43. **Ayllon J, Hale BG, Garcia-Sastre A.** 2012. Strain-specific contribution of NS1-activated phosphoinositide 3-kinase signaling to influenza A virus replication and virulence. J Virol **86:**5366-5370.

44. **Hrincius ER, Hennecke AK, Gensler L, Nordhoff C, Anhlan D, Vogel P, McCullers JA, Ludwig S, Ehrhardt C.** 2012. A single point mutation (Y89F) within the non-structural protein 1 of influenza A viruses limits epithelial cell tropism and virulence in mice. Am J Pathol **180:**2361-2374.

45. **Ehrhardt C, Wolff T, Ludwig S.** 2007. Activation of phosphatidylinositol 3-kinase signaling by the nonstructural NS1 protein is not conserved among type A and B influenza viruses. J Virol **81:**12097-12100.

46. **Patzina C, Botting CH, Garcia-Sastre A, Randall RE, Hale BG.** 2017. Human interactome of the influenza B virus NS1 protein. J Gen Virol **98:**2267-2273.

47. **Lopes AM, Domingues P, Zell R, Hale BG.** 2017. Structure-Guided Functional Annotation of the Influenza A Virus NS1 Protein Reveals Dynamic Evolution of the p85beta-Binding Site During Circulation in Humans. J Virol doi:10.1128/JVI.01081-17.

48. **Li W, Wang G, Zhang H, Shen Y, Dai J, Wu L, Zhou J, Jiang Z, Li K.** 2012. Inability of NS1 protein from an H5N1 influenza virus to activate PI3K/Akt signaling pathway correlates to the enhanced virus replication upon PI3K inhibition. Vet Res **43:**36.

49. **Shen YY, Liang L, Zhu ZH, Zhou WP, Irwin DM, Zhang YP.** 2010. Adaptive evolution of energy metabolism genes and the origin of flight in bats. Proc Natl Acad Sci U S A **107:**8666-8671.

50. **Seim I, Fang X, Xiong Z, Lobanov AV, Huang Z, Ma S, Feng Y, Turanov AA, Zhu Y, Lenz TL, Gerashchenko MV, Fan D, Hee Yim S, Yao X, Jordan D, Xiong Y, Ma Y, Lyapunov AN, Chen G, Kulakova OI, Sun Y, Lee SG, Bronson RT, Moskalev AA, Sunyaev SR, Zhang G, Krogh A, Wang J, Gladyshev VN.** 2013. Genome analysis reveals insights into physiology and longevity of the Brandt's bat Myotis brandtii. Nat Commun **4:**2212.

51. **Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, Fang X, Wynne JW, Xiong Z, Baker ML, Zhao W, Tachedjian M, Zhu Y, Zhou P, Jiang X, Ng J, Yang L, Wu L, Xiao J, Feng Y, Chen Y, Sun X, Zhang Y, Marsh GA, Crameri G, Broder CC, Frey KG, Wang LF, Wang J.** 2013. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science **339:**456-460.

52. **Campa CC, Ciraolo E, Ghigo A, Germena G, Hirsch E.** 2015. Crossroads of PI3K and Rac pathways. Small GTPases **6:**71-80.

53. **Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT.** 2017. The PI3K Pathway in Human Disease. Cell **170:**605-635.

54. **Biesold SE, Ritz D, Gloza-Rausch F, Wollny R, Drexler JF, Corman VM, Kalko EK, Oppong S, Drosten C, Muller MA.** 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat Eidolon helvum. PLoS One **6:**e28131.

55. **Eckerle I, Ehlen L, Kallies R, Wollny R, Corman VM, Cottontail VM, Tschapka M, Oppong S, Drosten C, Muller MA.** 2014. Bat airway epithelial cells: a novel tool for the study of zoonotic viruses. PLoS One **9:**e84679.

56. **Reuther P, Gopfert K, Dudek AH, Heiner M, Herold S, Schwemmle M.** 2015. Generation of a variety of stable Influenza A reporter viruses by genetic engineering of the NS gene segment. Sci Rep **5:**11346.

693 57. **Solorzano A, Webby RJ, Lager KM, Janke BH, Garcia-Sastre A, Richt JA.** 2005.
694 Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity
695 and confer attenuation in pigs. J Virol **79:**7535-7543.

696 58. **Anonymous.** The PyMOL Molecular Graphics System. Version 18 Schroedinger,
697 LLC.

698

699

Figure 1

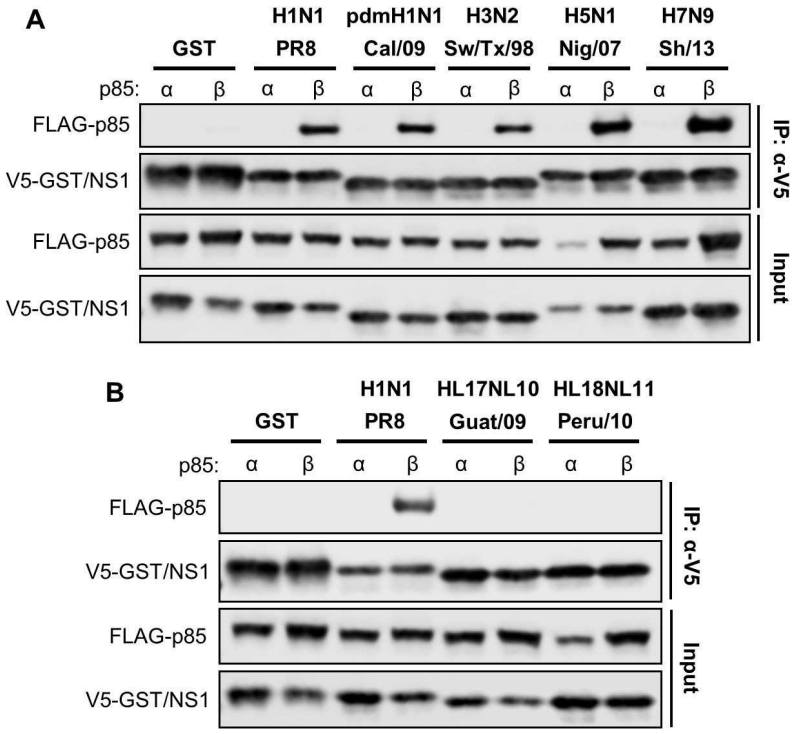
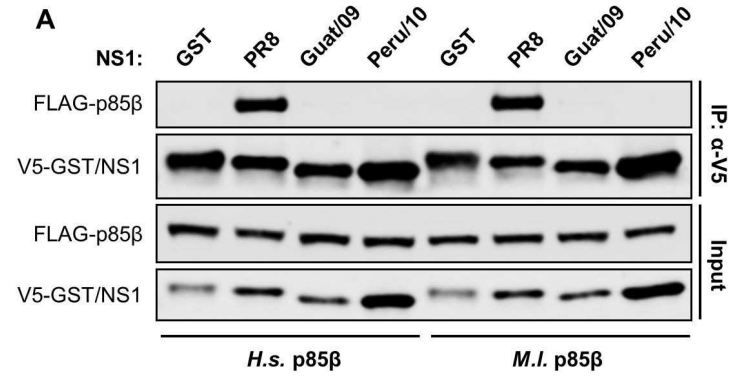


Figure 2



B

```
H. sapiens        DSVEAVGAQLKVYHQQYQDKSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQ 496
S. lilium         DSVEAVGAQLKVYHQQYQDKSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQ 495
M. lucifugus      DSVEAVGAQLKVYHQQYQDKSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQ 495
C. perspicillata  DSVEAVGAQLKVYHQQYQDKSREYDQLYEEYTRTSQELQMKRTAIEAFNETIKIFEEQGQ 495
                  ************************************************************

H. sapiens        TQEKCSKEYLERFRREGNEKEMQRILLNSERLKSRIAEIHESRTKLEQQLRAQASDNREI 556
S. lilium         TQEKCSKEYLERFRREGNEKEMQRILLNSERLKSRIAEIHESRTKLEQELRTQALDNREI 555
M. lucifugus      TQEKCSKEYLERFRREGNEKEMQRILLNSERLKSRIAEIHESRTKLEQELRTQALDNREI 555
C. perspicillata  TQEKCSKEYLERFRREGNEKEMQRILLNSERLKSRIAEIHESRTKLEQELRTQALDNREI 555
                  **************************************************:**:** *****

H. sapiens        DKRMNSLKPDLMQLRKIRDQYLVWLTQKGARQKKINEWLGIKNETEDQYALMEDEDDL 614
S. lilium         DKRMNSLKPDLMQLRKIRDQYLVWLTQKGARQKKINEWLGIKNETEDQYSLMEDEEDL 613
M. lucifugus      DKRMNSLKPDLMQLRKIRDQYLVWLTQKGARQKKINEWLGIKNETEDQYALMEDEDDL 613
C. perspicillata  DKRMNSLKPDLMQLRKIRDQYLVWLTQKGARQKKINEWLGIKNETEDQYSLMEDEDDL 613
                  ************************************************:*****:**
```

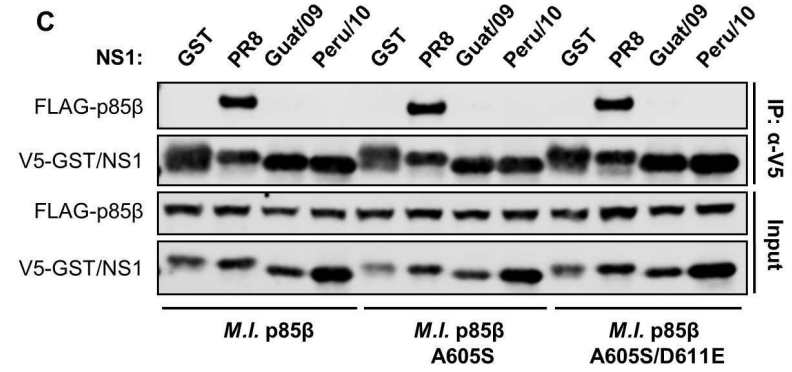


Figure 3

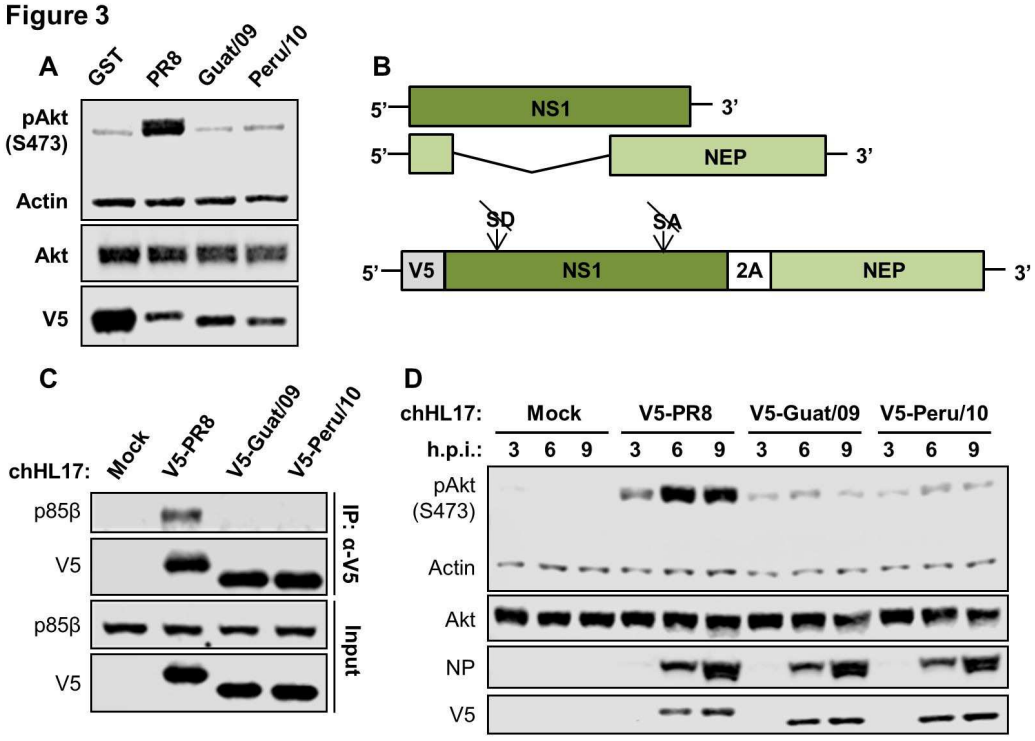


Figure 4

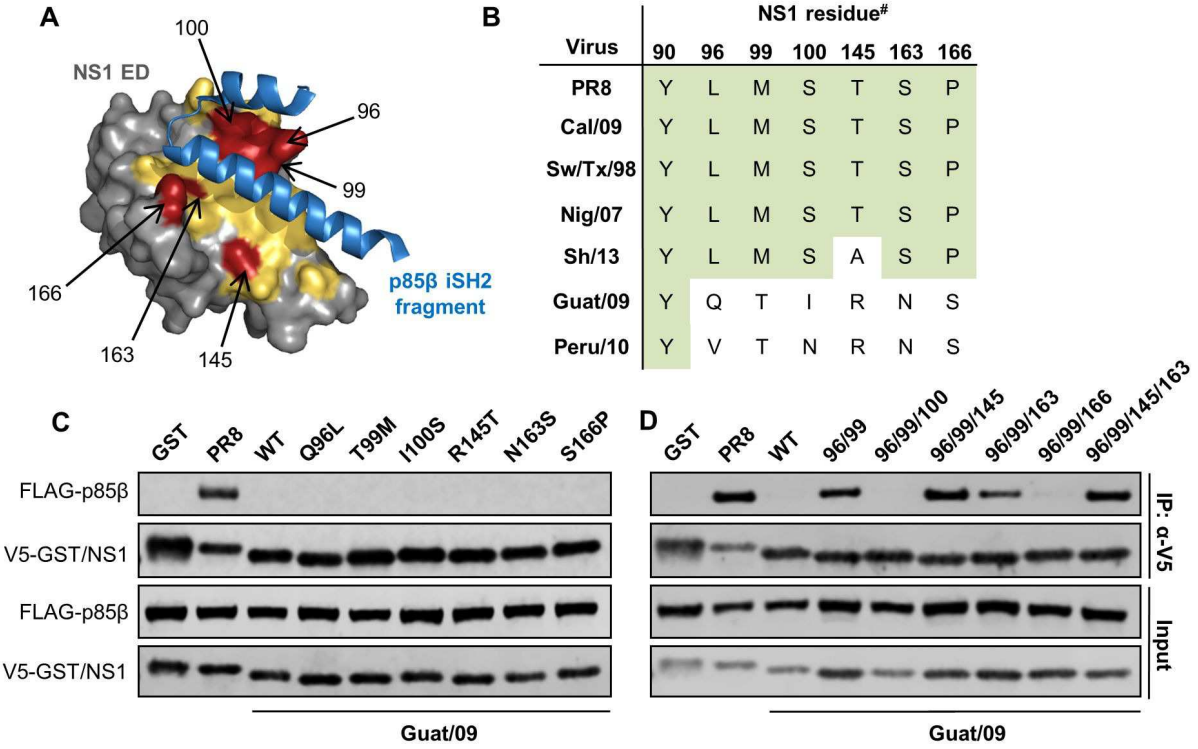


Figure 5

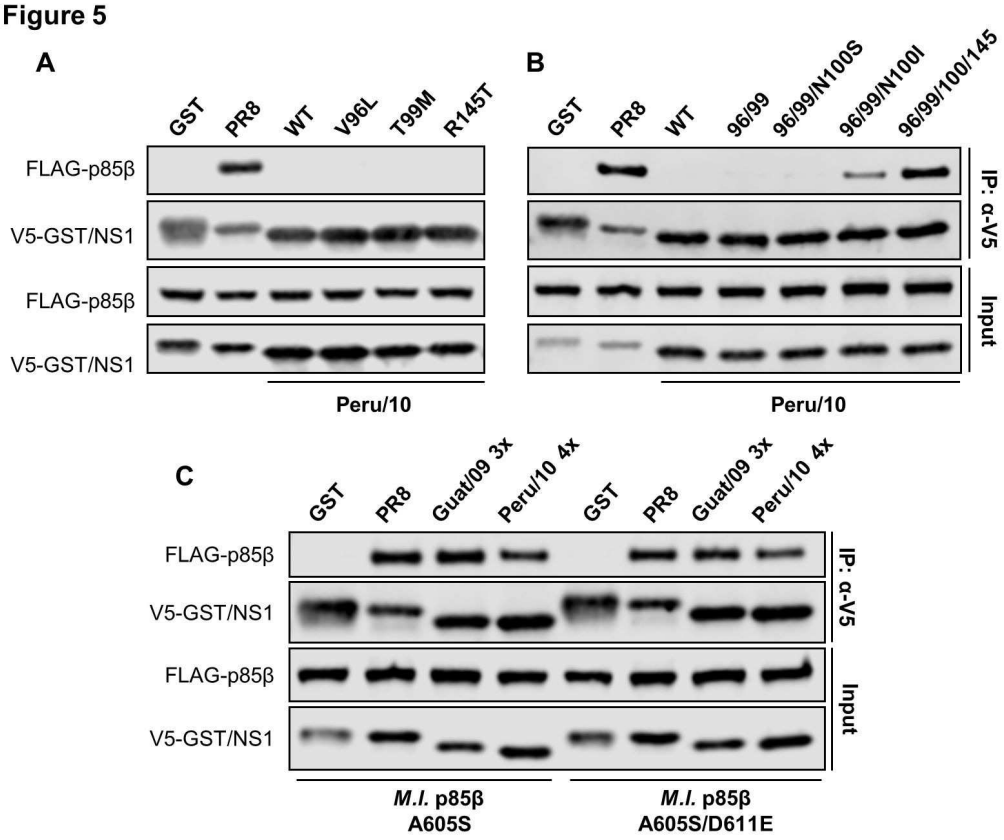


Figure 6

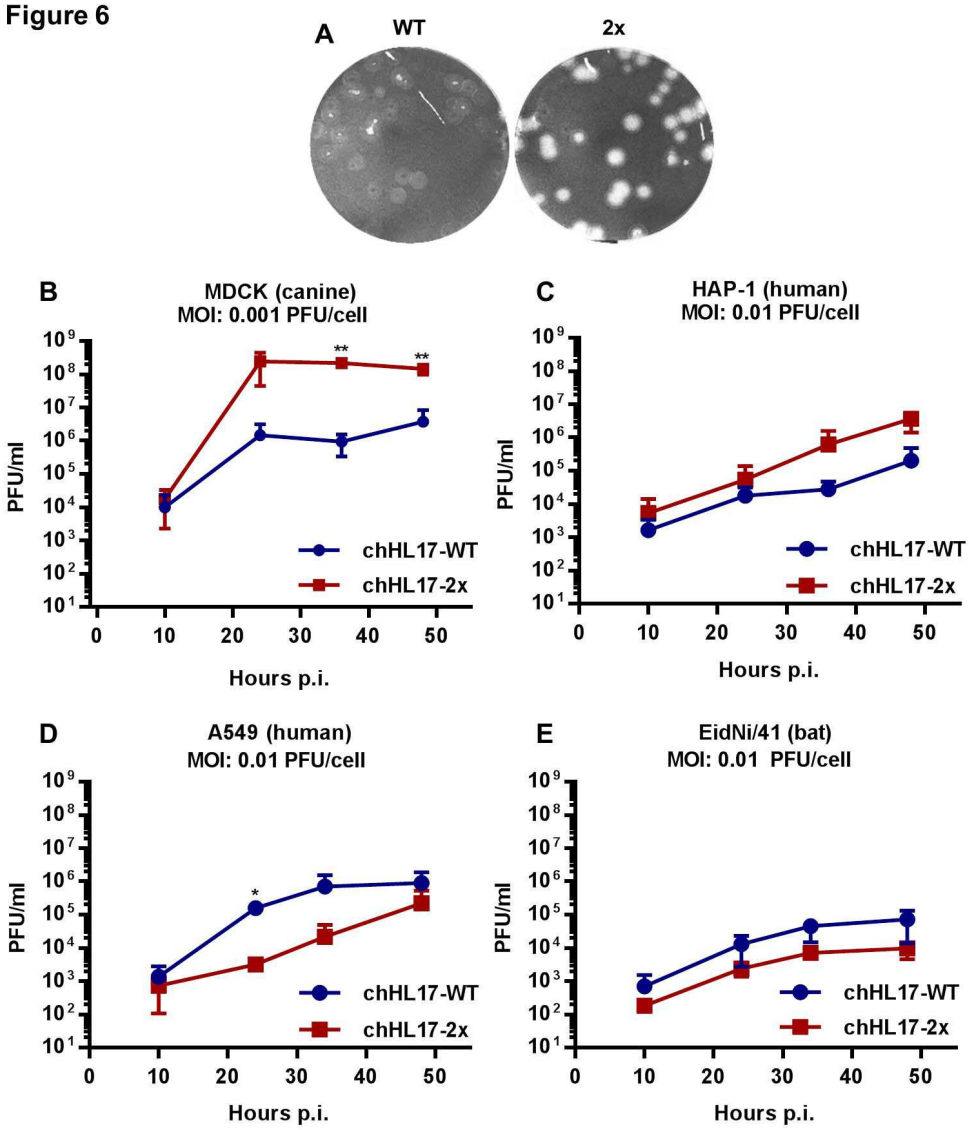


Figure 7